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Kinetics modeling of the acidolysis with immobilized *Rhizomucor miehei* lipases for production of structured lipids from sunflower oil

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ABSTRACT

Sunflower oil modification for production of semisolid fats was carried out via acidolysis using palmitic and stearic acids (P + St), hexane and a developed biocatalyst from *Rhizomucor miehei* lipases. Its kinetic behavior was studied by employing three mathematical models proposed in the literature. Furthermore, a new model was proposed to describe not only the variation of triacylglycerols (TAG), diacylglycerols (DAG), and free fatty acids groups but also the acyl migration reaction occurrence. The effect of the reaction temperature on the kinetic and equilibrium parameters, as well as TAG and reaction intermediates profiles was analyzed. Increasing reaction temperature generated major changes in the overall composition of acylglycerols and gave rise to the highest composition of P + St in the obtained structured lipids (58%, 70 h, 60 °C). P + St incorporation was successfully adjusted by an empirical model (Model I) and a lumped parameter model (Model II) for all the studied reaction times, while the model based on a Ping Pong Bi Bi mechanism (Model III) was only able to describe the kinetics behavior (through the variation of reactant saturated fatty acids concentration) until 24 h. Experimental data were fit satisfactorily by the proposed model (Model IV), showing that the increment in the disaturated TAG formation achieved by the increment in temperature was principally related to the favored DAG formation from triunsaturated TAG.

1. Introduction

Lipases can be used to obtain structured lipids (SL), which are tailor-made lipids with desired characteristics, like certain physical or chemical properties and/or nutritional benefits. Important structured triacylglycerols, like cocoa butter substitutes, low calorie fats, PUFA-enriched oils, and oleic acid enriched oils, have been synthetized by lipases [1]. Another important application is their use to convert oil into semi-solid fats without the formation of the undesired *trans* fatty acids. Among other processes, semi-solid fats can be synthesized by acidolysis reactions catalyzed by lipases, where it is possible to incorporate a desired acyl group onto a specific position of the triacylglycerol through the hydrolysis and reesterification reaction steps. In particular, by the use of *sn*-1,3 specific lipases as *Rhizomucor miehei*, free fatty acids (FFA) which are present in a reaction medium could be incorporated in *sn*-1 and *sn*-3 positions of triacylglycerols while original fatty acids are ideally kept in the *sn*-2 position. Following this route for the synthesis of SL, oils and saturated FFA have been used in order to obtain semisolid fats which are beneficial for human nutrition due to the fact that they preserve unsaturated or polyunsaturated long-chain fatty acids in the *sn*-2 position [2–5].

In previous works we studied the acidolysis of sunflower oil and a mixture of palmitic (P) and stearic (St) acids in a solvent medium in order to obtain semisolid fats. Lipases from *R. miehei* were immobilized on modified chitosan microspheres specially prepared to stabilize the open conformation of lipases and promotes their hyperactivation after their immobilization. They were used as a novel biocatalyst which, unlike some commercially available immobilized lipases, proved to be mechanically resistant for use in batch reactions when high agitation speeds were used. Moreover, it kept high activity for long reaction periods [6]. In order to understand the effect of the main reaction parameters, a response surface analysis and subsequently a multiresponse optimization with restriction based on practical considerations were performed. We found that under optimal working conditions after 24 h of reaction at 50 and 60 °C it was possible to achieve a change in the composition of palmitic and stearic acids from a value of 9.6% in the original oil to about 30% and 50% in the final SL, respectively [7].

In the present study we focused on the acidolysis kinetic, which is needed due to the lack of information about experimental

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Nomenclature

A, B, C	condensed kinetic parameters of Model II
E_T	enzyme concentration units (U)
F_M	fatty acids incorporation (mol of incorporated fatty acids/mol of total fatty acids in acylglycerides)
F_{Me}	fatty acids incorporation at equilibrium state (mol/mol)
IG	fatty acids in intermediate compounds (M)
k_j	apparent kinetic constant for the forward specific j reaction (1/(h M))
k_{-j}	apparent kinetic constant for the reverse specific j reaction (1/(h M))
K_e	equilibrium constant Model II
K_{II}	kinetic constant of Model III (1/M)
m_0	molar ratio of substrate, P + St/TAG (mol/mol)
m_e	biocatalyst mass (g)
p(LOF)	p-value of the lack of fit test
P_r	released fatty acids (M)
P + St	palmitic and stearic fatty acids
Q	new formed glyceride as product of the esterification (M)
$-r_S$	rate of disappearance of S in de reaction media (M/h)
S	reactant fatty acids (M)
[SUS]	molar concentration of the monounsaturated TAG (M)
[SSS]	molar concentration of the trisaturated TAG (M)
t	reaction time (h)
[UUU]	molar concentration of the triunsaturated TAG (M)
[$UU-$]	molar concentration of the diunsaturated DAG (M)
[$-US$]	molar concentration of the monounsaturated DAG (M)
V	reaction volume (L)
V_{R1}, V_{M2}	kinetic parameters of Model III (1/(h U))
W	water molar concentration (M)
W_0	initial water concentration as parameter of Model III and IV (M)
θ	treatment intensity parameter (g h/mol)

parameters of the reaction kinetics catalyzed by lipases and on modeling the behavior of the acidolysis reaction, especially between heterogeneous mixture of fatty acids and triacylglycerols [8–10]. Knowledge of this information is essential to control, optimize and design industrial reactors. Moreover, many studies concerning acidolysis reactions have been focused on the reaction products, without reporting other pertinent information such as concentrations of associated reaction media or reaction intermediates [11]. Consequently, not all of the available results about acidolysis reactions could be used to obtain kinetic parameters, especially when it is desired to employ a more sophisticated model [10]. In this contribution, the acidolysis reaction kinetic of sunflower oil with saturated fatty acids catalyzed by immobilized *R. miehei* lipases was studied employing mathematical models with different degrees of complexity. Three models found in literature were used to describe the saturated fatty acid incorporation to the original acylglycerols. Furthermore, a new approach to describe the variation over time of different triacylglycerol groups based on the saturation degree of their fatty acids was proposed. This takes into account the formation of diacylglycerols as main intermediate. Moreover, the occurrence of an acyl migration reaction was also considered to describe the appearance of trisaturated triacylglycerols. To the best of our knowledge, this last reaction has not been considered in other enzymatic acidolysis models. The effect of reaction conditions (temperature, time) on the kinetic and equilibrium parameters, as well as the formation of reaction intermediates (monoacylglycerols and diacylglycerols) was analyzed. On the other hand, the kinetic characteristics of free *R. miehei* lipase were studied at different temperatures with the objective of comparing its behavior with respect to the immobilized lipase.

To sum up, this contribution seeks to provide knowledge about the behavior of acidolysis reactions when a biocatalyst specially prepared for modifying lipids, is used. Rate expressions proposed in the literature were tested so as to verify its applicability to this specific system, which allowed obtaining new experimental values of kinetic constants under different conditions.

2. Materials and methods

2.1. Materials

Chitosan of low molecular weight with a degree of 75–85% of deacetylation and 12.6% of moisture was obtained from Sigma–Aldrich. Refined sunflower oil (SO) was purchased from a local grocery store and it was used as received (peroxide value (PV): 1.94 mequiv./kg). *R. miehei* lipase (>20,000 U/g, Novozymes) from *Aspergillus oryzae* conditioned in the form of liquid, dodecyl aldehyde (92%, Aldrich), sodium cyanoborohydride $NaCNBH_3$ (>95%, Fluka), palmitic acid–stearic acid mixture (P + St) – 98.9% purum with 53.0% of palmitic acid and 45.9% of stearic acid analyzed by GC – (Fluka) were purchased from Sigma–Aldrich (Germany). Fatty acid methyl esters (FAME) standards were purchased from Supelco (Bellefonte, USA). Other standards (1,2,3-trioctadecanoyl-glycerol, 1,2,3-trihexadecenoyl-glycerol, 1,2,3-trioctadecadienoyl-glycerol, 1,2,3-trioctadecenoyl-glycerol, 1,2,3-trihexa-decanoyl-glycerol, 1,2,3-tridecanoyl-glycerol, 1,2-distearoyl-3-palmitoyl-rac-glycerol, 1,3-dipalmitoyl-2-oleoylglycerol, 1,3-dioleoyl-2-palmitoyl-glycerol, 1,2-dilinoleyl-3-palmitoyl-rac-glycerol, 1,2-dioleoyl-3-stearoyl-rac-glycerol, 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol, 1,2-distearoyl-3-oleoyl-rac-glycerol, 1,3-dipalmitoyl-rac-glycerol, 1-monopalmitoyl-rac-glycerol, octadecenoic acid, tetradecane) were of more than 98% purity and were obtained from Sigma Chemical Co. (St. Louis, USA). Pyridine was from J.T. Baker (Philipsburg, USA) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Fluka (Buchs, Switzerland). All the other reagents, gases and solvents were of analytical or chromatographic grade.

2.2. Preparation of biocatalyst

Alkylated chitosan microspheres were prepared following the procedure described in our previous work [6], using dodecyl aldehyde in monomolar ratio (1:1 NH_2/aldehyde) and 1.5 h of contact time with aldehyde. Immobilization of lipases on the obtained support was carried out by adsorption as reported in the aforementioned work.

2.3. Lipase-catalyzed acidolysis reaction

2.3.1. Immobilized R. miehei lipase

Enzymatic acidolysis reactions of sunflower oil (SO) and a mixture of free palmitic and stearic acids (P + St) were carried out under the conditions previously determined as optimal from the operating standpoint [7]. Acidolysis was performed as follows: 110 mg of SO and 105 mg of P + St (1:3 molar ratio of substrates) were dissolved in 3.2 ml of hexane and later mixed and heated at desired reaction temperature. The reaction began when 340 mg of biocatalyst (immobilized lipase) was added. Reactions were performed in a screw-capped test tube in a water bath with temperature controller and magnetic agitation at 250 rpm. After a specified time, reactions were stopped removing biocatalyst by filtering. The solvent from

the reaction mixtures was removed by evaporation under a nitrogen atmosphere and then, the reaction mixtures were maintained at −20 °C until analysis.

In order to obtain the kinetic behavior, reactions were carried out at 50 and 60 °C, for reaction times from 1 to 70/72 h.

2.3.2. Free R. miehei lipase

Enzymatic acidolysis reactions of sunflower oil and a mixture of P + St catalyzed by free *R. miehei* lipase were carried out under temperature and substrate ratio conditions previously determined as suitable for the enzyme. The reaction mixture consisted in 400 mg of SO and the required quantity of P + St and hexane. The procedure was continued as it was described above, but adding 80 μl (4.42 mg of protein) of commercial lipase preparation to the reaction medium to initiate the reaction. After the specified reaction time, 2 ml of acetone were added to precipitate proteins [12], followed by filtration with sodium sulfate in order to remove them. Reaction times assayed were 2, 4, 8 and 24 h at three different temperatures: 30, 40 and 50 °C.

2.4. Acidolysis products analysis

2.4.1. Fatty acid composition

Acidolysis reaction products were purified by alkaline deacidification in order to remove free fatty acids, as suggested by Carrín and Crapiste [13]. FAME from SL were prepared by cold transesterification with methanolic KOH according to the Official Method Ce 2-66 [14], and were analyzed by gas–liquid chromatography (GLC) with a 4890D series gas chromatograph (Agilent, Hewlett-Packard) and a fused-silica capillary column (SP-2380, 30 m × 0.25 mm × 0.2 m film thickness; Supelco Inc.). The carrier gas was hydrogen with a linear velocity of 18 cm/s. The injector was used in split mode with a ratio of 1:50. The oven temperature was programmed to be at 170 °C for 15 min, further increased to 210 °C at a rate of 4 °C/min, and held for 10 min. The injector and detector temperatures were 220 °C. FAME were identified by comparing their retention times with authentic standards. Data acquisition and peak integration were performed using HP 3398A GC Chemstation Software (Hewlett-Packard, 1998).

2.4.2. Triacylglycerol (TAG) profiles

TAG quantification of the non-deacidified reaction product was performed by GLC by means of a 4890D series gas chromatograph (Agilent, Hewlett-Packard) equipped with a FID (adapted from IRMM Method EUR 20831 EN). A metallic capillary column (MXT- 65TG, 30 m × 0.25 mm × 0.1 m film thickness; Restek, Bellefonte, USA) was used. The injector was used in split mode (split ratio of 1:70) and held at 360 °C. The detector temperature was constant and equal to 380 °C. The oven temperature was programmed to be at 40 °C for 4 min, then increased first from 40 °C to 350 °C at the rate of 15 °C/min and then to 360 °C at the rate of 0.2 °C/min. Hydrogen was used as the carrier gas at a linear velocity of 33.6 cm/s. Internal standard method was used to quantify TAG using tripalmitolein as standard. Relative response factors of all available standard TAG were correlated with their relative residence time in order to quantify TAG whose standards were not available. Data acquisition and peak integration were performed using HP 3398A GC Chemstation Software (Hewlett-Packard, 1998). Where necessary, the identified TAGs were grouped in four different categories according to the number of saturated (S) and unsaturated (U) fatty acids in the molecule (without distinguishing positional isomers). Consequently, content of TAG in products can be reported as SSS, SUS (including its isomers SSU and USS), UUS (including its isomers SUU and USU), and UUU (% g/100 g on total TAG).

2.4.3. Reaction intermediates analysis

FFA, monoacylglycerols (MAG) and DAG were analyzed in simultaneous mode with TAG analysis by GLC. This technique also allowed us to identify the presence of free glycerol in the analyzed sample. The internal standard method was used to quantify each group of by-products (FFA, MAG, and DAG) with a calibration curve for each one, being tetradecane the internal standard for FFA and glyceryl tridecanoate for MAG and DAG. Calibration curves were constructed using oleic acid, monopalmitin, and dipalmitin as standards of FFA, MAG, and DAG, respectively. Data acquisition and peak integration were carried out using HP 3398A GC Chemstation Software (Hewlett-Packard, 1998).

2.5. Mathematical modeling of the acidolysis reaction kinetics

2.5.1. Background and general considerations

Without a doubt, the most widely used equation over time to describe enzyme kinetics has been the Michaelis–Menten equation. This has been used to represent adequately numerous enzymatic reactions which involve a single reactant and a homogeneous system in the initial stage of reaction [15–18]. However, more complex reactions involving two or more reactants such as the enzymatic acidolysis reaction, in which also it takes place in a heterogeneous system (lipid–water interface), can not be described by Michaelis–Menten equation and its variants because they do not fulfill the conditions specified above. In these, the complexity is presented by a way in which substrates and products are bonded and unbonded from active site. This gives rise to various mechanisms that can generate different expressions for the reaction rate, which affect the accuracy of the generated models [19,20].

Enzyme kinetics for acidolysis reaction have been usually described by the Ping Pong Bi Bi mechanism, which is applicable to lipase reactions in several media types [21,22]. Balcão and Malcata [23] presented the mathematical modeling of this mechanism applied to a generic lipase-catalyzed reaction and the associated rate expression assuming pseudo-stationary state. That modeling can be simplified depending on the kinetic assumptions, but usually at least four parameters are involved. Furthermore, Reyes and Hill [24] used a Ping Pong Bi Bi mechanism in a simplified form to model the dynamic behavior of acidolysis reactions in the absence of solvent. They obtained two expressions that require knowledge of a total of five kinetic parameters and the pursuit of concentrations of five components over time. Then, Ortega et al. [25] applied that model to represent successfully the incorporation of conjugated linoleic acid in fully hydrogenated soybean oil in a system with solvent. On the other hand, Xu et al. [26] proposed a simplified model for the kinetic of acidolysis reactions in solvent-free system where the incorporation was subject to reaction time in a similar manner as the reaction rate does with the substrate concentration in the Michaelis–Menten equation. Besides, Camacho et al. [11] postulated a lumped parameter kinetic model for acidolysis catalyzed by a *sn*-1,3 specific immobilized lipase in a solvent system. That model requires knowledge of three experimental parameters and only one variable over time. The three abovementioned models were used by Pacheco et al. [10] to describe the kinetic behavior of acidolysis reaction between sunflower oil and saturated fatty acids using a commercial immobilized lipase (Lipozyme RMIM). They found that the proposed models fit successfully the experimental data. Recently, Ray et al. [27] described the kinetic behavior of an immobilized *Rhizopus oryzae* lipase during the acidolysis of high oleic sunflower oil with palmitic and stearic acids. They proposed a first order reactions scheme taking into account all possible combinations to form TAG and DAG, preserving the unsaturated fatty acid in their *sn*-2 position.

2.5.2. Kinetic models

Firstly, this section describes briefly the different kinetics models suitable for enzymatic acidolysis reactions that were found in the literature, which were used to fit the experimental data. It is only shown the mathematical expression associated to each model and assumptions performed by the authors to develop them. More detailed information can be found in the mentioned references.

Additionally, a kinetic model based on the possible reversible reactions involved between different TAG groups, FFA and DAG catalyzed by the 1,3-positional specific lipase was developed. Moreover, it takes into account the possibility of an acyl migration reaction occurrence based on the appearance of trisaturated triacylglycerols.

2.5.2.1. Kinetic Model I. The simplest model available in the literature to represent the acidolysis reaction through incorporation of free fatty acids (FFA) with the reaction time is the model proposed by Xu et al. [26].

$$F_M = \frac{F_{Me} \cdot t}{t_{1/2} + t} \quad (1)$$

where F_M is the incorporation of reactants fatty acids into the glycerol backbone, F_{Me} is the maximum F_M corresponding to equilibrium and $t_{1/2}$ is the time required to reach the half of the F_{Me}. Both parameters are determined experimentally. This model is empirical and it is not associated with any hypothesis or simplification, and the incorporated fatty acids are those fatty acids that were reactive and now are present in the acylglycerols, regardless of their positions on the glycerol backbone.

2.5.2.2. Kinetic Model II. A lumped parameter kinetic model for the acidolysis of triacylglycerols (TAG) and free fatty acids (FFA) in non-aqueous media, catalyzed by a *sn*-1,3 specific immobilized lipase was proposed by Camacho et al. [11]. This model was developed based on the mechanism of the acidolysis reaction by considering the following hypothesis: (1) due to the positional specificity of the lipase, only fatty acids in *sn*-1,3 positions of the TAG are involved in the reaction; (2) the substitution in one position is independent of the nature of the fatty acid present in the other possible position to be substituted, namely both positions are equivalents; (3) diacylglycerols (DAG) concentration reaches stationary state in short reaction time and remains at low level; (4) acyl-enzyme complexes are the only intermediates with appreciable life where enzyme participates, being in equilibrium with FFA; (5) acyl-enzyme concentrations are large with respect to free enzyme concentration.

The model represents the variation of the fatty acids incorporated to acylglycerols, expressed in molar fraction through F_M variable, with the treatment intensity θ as a function of kinetic and equilibrium parameters and F_M in itself. The reduced form of the model is:

$$\frac{dF_M}{d\theta} = \frac{A[(2-3F_M)(m_0-3F_M) - (1/K_e)(3F_M)^2]}{[B(3F_M)+(m_0-3F_M)][(BC)(3F_M)+(m_0-3F_M)]} \quad (2)$$

where θ is defined in terms of the biocatalyst mass (m_e), reaction volume (V), initial concentration of triacylglycerols ($[TAG]_0$) and reaction time (t), as:

$$\theta = \left(\frac{m_e}{V[TAG]_0}\right) t \quad (3)$$

The equilibrium constant (K_e) is obtained based on the F_M value in steady state conditions (F_{Me}), according to the following:

$$K_e = \frac{(3F_{Me})^2}{(2-3F_{Me})(m_0-3F_{Me})} \quad (4)$$

where the condensed kinetic parameters A, B and C, were chosen taking into account the meaning of the lumped variables. $A = k_{-L}E_T/(3K_M)$ (relation between formation kinetic constant of acyl-enzyme complex from original TAG (k_{-L}), active enzyme concentration (E_T) and equilibrium constant of the acyl-enzyme complex formation from free reactant fatty acids (K_M)), $B = K_L/K_M$ (relation between equilibrium constants of the acyl-enzyme complexes formation from free released/reactant fatty acids) and $C = k_L/k_M$ (relation between DAG esterification kinetic constants with fatty acids of the triacylglycerols original (k_L)/fatty acid reactants (k_M)).

2.5.2.3. Kinetic Model III. Reyes and Hill [24] postulated three different mechanisms for the acidolysis between free fatty acids and a heterogeneous triacylglycerol, such as olive oil or milk fat, differing from one another in the point where the water molecule enters the catalytic cycle. The expressions developed by the authors take into account the effect of the concentration of all chemical species participating in the reaction, resulting in more complex models than those previously described. Assumptions carried out in the models development were the followings: (1) all the fatty acids released from TAG substrate can be lumped together as a single product (Pr); (2) the lowest acylglycerols species (glycerol, mono- and diacylglycerols) can be lumped together in a single intermediate compound, whose fatty acid composition is IG; (3) the rate determining step is the one that involves the rupture of the ester bond, assuming that the other steps reach a rapid equilibrium.

Here, the authors used as variables to be tested the rate of appearance of fatty acids in the products (r_{Pr}) and the rate of disappearance of free fatty acids ($-r_S$), rather than fatty acids incorporation like the previous models. Each of the three models developed by Reyes and Hill [24], derived from each proposed mechanism, were tested in the setting of experimental data obtained in free solvent acidolysis reaction and it was found that the reduced version of the rate expression corresponding to their mechanism number 1 (in which water enters the catalytic cycle during acylation/deacylation steps), resulted in the best fit:

$$-r_S = \frac{(V_{R1}[S][IG] - V_{M2}[W][Q])E_T}{(1+K_{II}[IG])([S]+[P_r])} \quad (5)$$

where V_{R1}, V_{M2} and K_{II} are kinetics parameters. It is worth mentioning that Reyes and Hill [24] found differences between calculated and theoretical initial water content necessary to achieve levels of hydrolysis observed after each acidolysis reaction. Accordingly, the authors suggested considering the initial concentration of water (W_0) as an additional parameter to incorporate into the model.

2.5.2.4. Kinetic Model IV: proposed kinetic model. During acidolysis reactions catalyzed by enzymes a huge number of different chemical reactions take place involving not only substrates and products but also enzymatic intermediates. So, it is difficult to obtain a detailed description of the evolution of all the involved components [24]. In this work, a simple mathematical model based on the behavior of TAG, DAG, and FFA groups according to their saturated and unsaturated fatty acids composition, was developed following these assumptions: (1) based on the specificity characteristics of the used lipase, only fatty acids in *sn*-1,3 positions of the TAG are involved in the enzymatic reactions; (2) *sn*-1 and *sn*-3 positions of TAG are equivalents; (3) the enzyme does not show preference for a specific fatty acid; (4) trisaturated TAG (SSS) can be obtained due to acyl-migration as side reaction; (5) all reactions can be described by first order kinetics; (6) immobilized enzyme deactivation is not considered taking in consideration the high stability showed by these biocatalysts in previous results [6]; (7) MAG formation is not considered due to the low levels obtained during the progress of the acidolysis reaction; (8) mass transfer resistances are not considered since the reactor was well mixed and the enzymes were adsorbed on the support surface [6,9,27].

Condensed reactions involved in the proposed mechanism are:

$$UUU + W \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} UU- + U \quad (6)$$

$$UU- + S \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} UUS + W \quad (7)$$

$$UUS + W \underset{k_{-3}}{\overset{k_3}{\leftrightarrow}} -US + U \quad (8)$$

$$-US + S \underset{k_{-4}}{\overset{k_4}{\leftrightarrow}} SUS + W \quad (9)$$

$$SUS + S \xrightarrow{k_5} SSS + U \quad (10)$$

Although it is not explicitly showed in this condensed reaction scheme, Eqs. (6)–(9) involved enzymatic catalysis, whereas Eq. (10) is considered a non-enzymatic reaction.

The set of ordinary nonlinear differential equations derived from the above reaction mechanism is:

$$\frac{d[UUU]}{dt} = -k_1[UUU][W] + k_{-1}[UU-][U] \quad (11)$$

$$\frac{d[UU-]}{dt} = k_1[UUU][W] - k_{-1}[UU-][U] - k_2[UU-][S] + k_{-2}[UUS][W] \quad (12)$$

$$\frac{d[UUS]}{dt} = k_2[UU-][S] - k_{-2}[UUS][W] - k_3[UUS][W] + k_{-3}[-US][U] \quad (13)$$

$$\frac{d[-US]}{dt} = k_3[UUS][W] - k_{-3}[-US][U] - k_4[-US][S] + k_{-4}[SUS][W] \quad (14)$$

$$\frac{d[SUS]}{dt} = k_4[-US][S] - k_{-4}[SUS][W] - k_5[SUS][S] \quad (15)$$

$$\frac{d[SSS]}{dt} = k_5[SUS][S] \quad (16)$$

$$\frac{d[U]}{dt} = k_1[UUU][W] - k_{-1}[UU-][U] + k_3[UUS][W] - k_{-3}[-US][U] + k_5[SUS][S] \quad (17)$$

$$\frac{d[S]}{dt} = -k_2[UU-][S] + k_{-2}[UUS][W] - k_4[-US][S] + k_{-4}[SUS][W] - k_5[SUS][S] \quad (18)$$

$$\frac{d[W]}{dt} = -k_1[UUU][W] + k_{-1}[UU-][U] + k_2[UU-][S] - k_{-2}[UUS][W] - k_3[UUS][W] + k_{-3}[-US][U] + k_4[-US][S] - k_{-4}[SUS][W] \quad (19)$$

where $[i]$ is the molar concentration of the i species, UUU = triunsaturated TAG, UUS = diunsaturated TAG (including its isomers SUU and USU), SUS = monounsaturated TAG (including its isomers SSU and USS), SSS = trisaturated TAG, UU− = diunsaturated DAG, −US = monounsaturated DAG, W = water, S = free saturated fatty acids, U = free unsaturated fatty acids, k_j and k_{-j} = apparent kinetic constant for the forward and reverse specific j reaction, respectively, related to Eqs. (6)–(10), and t = reaction time. Initial water concentration (W_0) associated to Eq. (19) is a parameter to be determined by solving the differential equation system. It is worth mentioning that the effect of the enzyme concentration would be implicitly included in these apparent kinetic constants, except in k_5.

2.6. Statistical analysis

Reactions were done by duplicate and each sample was analyzed twice generating four values of the same analyses. Statistical differences between average values were evaluated with the t-test. A confidence value of 95% was used. Results are shown as average value of four results ± standard deviation. When the variation coefficient between two replicates was higher than 10%, a third independent replicate was made to disregard the outlier.

Experimental data were fitted with the models I, II, and III using linear and nonlinear algorithms contained in Origin Pro8 (OriginLab, 2007) and Systat (Systat 13, 2009) programs. Kinetic equations of the proposed Model IV (Eqs. (11)–(19)) were specified in SimBiology (a MatLab toolbook) and associated parameters were obtained using a nonlinear least square optimization method performed by the 'lsqcurvefit' function of MatLab 7.5.0 (2007).

3. Results and discussion

3.1. Sunflower oil composition

The approximate fatty acids composition of the original SO, obtained by GLC analysis as fatty acid methyl esters (FAME), was: 56.6% C18:2 (linoleic acid (L)), 31.0% C18:1 (oleic acid (O)), 6.3% C16:0 (palmitic acid (P)) and 3.2% C18:0 (stearic acid (St)). As expected, the SO was abundant in unsaturated fatty acids and the FA composition was in agreement with other studies [28–30]. The approximate acylglycerols composition, obtained by GLC analysis, was: 0.11% monoacylglycerols, 1.04% diacylglycerols and 98.85% triacylglycerols. The TAG composition (g/100 g) in SO was: POP = 0.30 ± 0.00, PLP = 0.98 ± 0.02, StStP = 0.07 ± 0.01, POSt = 0.29 ± 0.01, POO = 2.80 ± 0.03, PLSt = 0.85 ± 0.00, POL = 6.54 ± 0.07, PLL = 8.30 ± 0.13, StStSt = 0.04 ± 0.01, StStO = 0.13 ± 0.03, OOSt = 1.26 ± 0.01, OOO = 8.12 ± 0.00, StOL = 2.59 ± 0.43, OOL = 15.23 ± 0.30, StStL = 3.06 ± 0.14, OLL = 27.36 ± 0.06, LLL = 21.81 ± 0.51. A chromatogram of the oil TAG profile is shown in Supplementary Fig. 1A. As it was stated above, it must be taken into mind that used nomenclature is not strictly related to a specific position isomer of the corresponding TAG.

3.2. Acidolysis with free R. miehei lipases

Fig. 1 shows the amount of palmitic and stearic fatty acids incorporated to original triacylglycerols by the action of free *R. miehei* lipases at different temperatures. As it can be observed, the maximum incorporation occurs at 40 °C, according to the value reported in the literature as the optimal temperature for this lipase [31]. After 24 h of reaction, nearly 40% of saturated fatty acids were

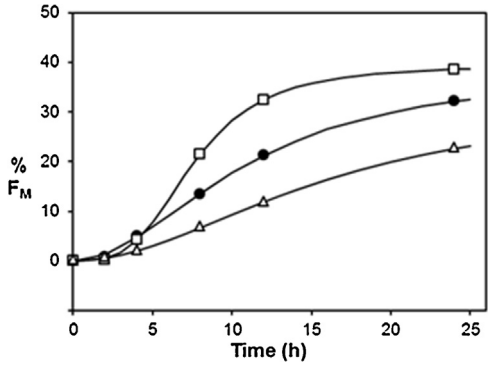

Fig. 1. Incorporation of P + St into SO during acidolysis reaction using free lipase as a function of time and temperature (30 °C = ●, 40 °C = □ and 50 °C = △): fitting of experimental data by kinetic Modified Model I (–).

incorporated into acylglycerols by the enzyme. At 30 °C, the reaction time course followed a similar trend to the reaction carried out at 40 °C but it showed a lower final incorporation level; while at 50 °C the acidolysis seemed to present a more linear relationship with time showing the lowest final incorporation level. Likewise it can be noted that, at shorter times, there is a period in which considerable incorporation was not obtained in the three tested temperatures, contributing to the sigmoidal form of the obtained curves. In attempting to portray this behavior using the Model I (Eq. (1)), generated curves failed to properly fit to experimental points. For this reason, it was necessary to look for an equation that could adequately describe this behavior. The so-called Hill equation [32], used for allosteric enzymes, can be used in these cases, replacing the speed of reaction and substrate concentration with free fatty acids incorporation and time, respectively. This is a modified form of Model I which incorporates an additional constant, n.

$$F_M = \frac{F_{Me} \cdot t^n}{t_{1/2}^n + t^n} \quad (20)$$

where F_{Me} is the reactants fatty acids incorporation corresponding to equilibrium state, and $t_{1/2}$ and n are constants to be determined experimentally.

When Modified Model I was applied, the description of experimental data with an excellent level of correlation was achieved, as shown in Fig. 1. Values for the parameters of this model, F_{Me}, $t_{1/2}$ and n, and the determination coefficient (R^2) associated with the model are shown in Table 1.

The existence of a period in which no incorporation of fatty acids takes place at first hours of reaction was also observed by Camacho et al. [9] who called it the "lag period". They employed commercial immobilized lipase of *R. miehei* (Lipozyme IM) in a packed bed reactor for the acidolysis reaction between cod liver oil and caprylic acid. They found that the lower the water content of the biocatalyst, the greater the duration of the lag period. They suggested that this time is required to reach the equilibrium water content of a hydroenzymatic layer that surrounds the support. This layer is formed by the water of saturation contained in the solvent and the water consumed by the formation of a small amount of diacylglycerols. Taking this into account, and considering that the lipases used in this work were in their soluble state, it seems logical to think that during the lag period lipases interacted with water molecules from the medium prior to show enzymatic activity. Thus, the formation of a hydration layer which served as the primary component of the enzyme microenvironment in the organic medium could be taking place. In general, it may be thought that free lipases would be adapting to the new media during this lag period. Once lipases were hydrated and reached their active conformation their catalytic power was increased accelerating the reaction rate [33]. Water introduced into the system came mainly from commercial lipase preparation which contains about 50% of water, as reported by Bjurlin et al. [34]. Since conditions under which reactions were carried out were the same – only temperature and time were varied – it is expected that all the studied systems have had the same water content. However, changes in temperature could influence the thermodynamic properties of the system.

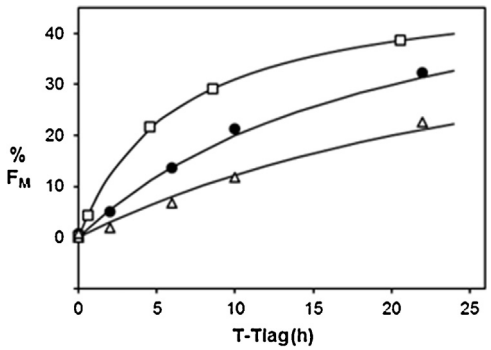

Fig. 2. Incorporation of P + St into SO during acidolysis reaction using free lipase as a function of time, subtracting the lag period, and temperature (30 °C = ●, 40 °C = □ and 50 °C = △): fitting of experimental values by kinetic Model I (–).

The parameter associated with the duration of the lag period in Eq. (20) is n. Increasing of n is due to the increase of the length of inactivity time, while that $n = 1$ implies that the phase lag does not exist. Furthermore, this parameter also affects final incorporation value and reaction rate that follow to lag period. When the adjustment was made, it was found almost the same value of n for temperatures of 30 and 50 °C, but at 40 °C differed from previous ones. This was probably due to the fact that maximum and minimum temperature tested exhibited analogous behavior in the pseudo-initial reaction rates, although the predicted equilibrium incorporations were not similar, it which generate globally a compensation on the obtained n value.

Experimental points can be fitted with Model I after correcting these by subtracting the times corresponding to the lag period (n hours). In this case, curves plotted in Fig. 2 with the associated parameters F_{Me} and $t_{1/2}$ given in Table 2 were obtained. Based on the determination coefficient value, the proposed model fits satisfactorily to experimental data.

When comparing fitting parameters of both models, it can be observed that the Modified Model I predicts lower levels of incorporation for the equilibrium (F_{Me}) than the Model I. On the other hand, $t_{1/2}$ values were similar in each temperature, except for $T = 50$ °C. Thus, it is evident that models represent satisfactorily the actual behavior on time ranges in which they were defined.

The effect of temperature on the free lipases activity in the acidolysis reaction can be better appreciated when the percentage of saturated fatty acids incorporation ($\%F_M$) after 24 h of reaction is represented (Fig. 3). The highest incorporation was achieved at the optimum temperature of the enzyme. In decreasing order of activity it was: 40 > 30 > 50 > 60 °C. Lower activities found at higher tested temperatures were associated with partial inactivation of free lipases [35].

As far as we know, all previous works that have analyzed temperature effect on acidolysis reaction catalyzed by *R. miehei* lipases have used immobilized lipases [10,36,37]. Immobilization processes usually generate enzymatic derivatives more thermally stable than free lipases. Therefore, it was not possible to make a comparison or validation of the profile found. Yang et al. [38] showed the shift of the temperature vs. activity curve toward a higher value of optimum temperature when immobilized

Table 1
Parameters of Modified Model I (Eq. (20)) for acidolysis with free *Rhizomucor miehei* lipases at assayed reaction temperatures and corresponding determination coefficients.

T (°C)	F_{Me} (mol%)	$t_{1/2}$ (h)	n	R^2
30	38.97	10.926	1.982	0.9975
40	39.42	7.570	3.360	0.9997
50	32.72	15.945	1.973	0.9996

Table 2
Parameters of Model I (Eq. (1)) for acidolysis with free *Rhizomucor miehei* lipases at assayed reaction temperatures and corresponding determination coefficients.

T (°C)	F_{Me} (mol%)	$t_{1/2}$ (h)	R^2
30	46.84	10.001	0.9881
40	50.27	6.194	0.9999
50	40.85	19.075	0.9915

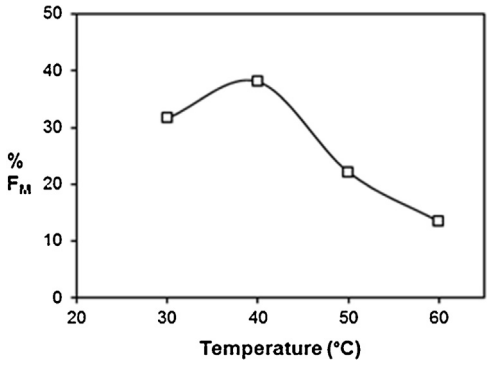

Fig. 3. Effect of reaction temperature in acidolysis using free *Rhizomucor miehei* lipases.

derivatives of *Candida antarctica* lipase were used instead of its free form during the olive oil hydrolysis. This study also showed a similar behavior for the free enzyme to that reported in Fig. 3, although being another type of lipase.

3.3. Acidolysis with immobilized R. miehei lipases

3.3.1. Composition of palmitic and stearic acids in structured lipids

The evolution in time of palmitic and stearic acids composition (P + St) in the structured lipids at 50 °C and 60 °C is shown in Supplementary Fig. 2. Significant differences in fatty acids incorporation due to the temperature were found after 4 h of reaction, in which at 60 °C it was markedly faster. After 70 h of reaction at 60 °C, the obtained structured lipids (SL) contained about 58% of P + St in acylglycerols. After a similar time (72 h) at 50 °C, SL contained about 42% of P + St. This positive effect on immobilized lipases activity at higher temperature has also been reported in previous works by commercial biocatalysts from *R. miehei* [10,13,39].

3.3.2. Composition of acylglycerols in structured lipids

As it is known, the acidolysis mechanism is initiated by hydrolysis of original fatty acids which allows subsequent esterification of acylglycerols with new acyl chains. Hydrolysis products, free fatty acids, monoacylglycerols, and diacylglycerols, are necessary intermediates. Since the concentration of free fatty acids remains practically constant during the reaction, factors analyzed were variations over the time of remaining components together with triacylglycerols (see Supplementary Fig. 3A). As it can be observed for both reaction temperatures, during acidolysis reaction diacylglycerols and monoacylglycerols were generated (this last one in a considerably lesser proportion), which led to the decrease of triacylglycerols. Most important changes in the compositions of triacylglycerols and diacylglycerols were observed from the start of the reaction until about 12 h and then gradually were stabilized up to reach different stationary states. With regards to monoacylglycerols, although its composition was very low over the analyzed time range, by amplifying the scale (see Supplementary Fig. 3B) it is appreciated that this content does not seem to stabilize at 60 °C, because they continued to increase. Likewise, this analysis showed that the increased reaction temperature generated major changes in the overall composition of acylglycerols. Moreover, it could be said that the hydrolysis step was favored over the esterification one with the increase in temperature from 50 to 60 °C.

Proportional changes in TAG types over time and temperature could be observed in Supplementary Fig. 4. Triunsaturated TAG (UUU, majority in the original oil) decreased over time, with the complement increment of other TAG types (specially disaturated types SUS). The effect of temperature could be appreciated there, since at 60 °C a reduction about 50% of UUU was obtained after 10 h of reaction whereas at 50 °C it took nearly 70 h to reach a similar level. Moreover, the behavior of diunsaturated TAG (UUS) differed with the change of temperature. UUS reached a maximum near 24 h at 60 °C, after which its proportion in the TAG fraction decreased. On the other hand, at 50 °C the higher value reached was maintained from 24 until 70 h of reaction. It is worth mentioning observed changes of trisaturated TAG (SSS). Its growing presence on the TAG fraction is closely related to the evolution of acyl migration reactions since the used lipase is known as *sn*-1,3 specific one. It could be seen that the migration was more favored at 60 °C than at 50 °C. For instance, when the reaction took place at 50 °C and 60 °C after approximately 70 h, the SSS group was 13.82% and 20.89%, respectively.

Regarding the SUS group, we wanted it to be the major reaction product, since these lipids contain two saturated fatty acids that give features of solid or semisolid fats. Furthermore, the SUS group would preserve the unsaturated fatty acid in *sn*-2 position, provided that the enzyme is specific and migration of the original unsaturated FA from the *sn*-2 position to the outside positions does not occur. As it can be observed, at 60 °C this group increased approximately linearly until 12 h. After this time, the rise progressed at a slower rate, until becoming the majority group and representing approximately 40% of total TAG after 70.5 h of reaction. It is worth mentioning that, from the chromatographic analysis used, we are not able to assure that this group of monounsaturated TAG is exclusively of the SUS type (with the unsaturated fatty acid in its *sn*-2 position). However, from results obtained analyzing the fatty acid composition of the glycerides *sn*-2 position, only 10% of the total saturated fatty acids found in that position could not be related to the original oil composition and the formation of SSS (data not shown). So, although the SUS group would not have exclusively an unsaturated fatty acid in its *sn*-2 position, this would be its most probable occurrence. On the other hand, at 50 °C this group was increased until it reached a value of 30% of total TAG after 72 h of reaction.

Twenty different species of triacylglycerols were identified in the reaction products (see Supplementary Fig. 1B), the species found in SO as well as PPP, PPSt, and StLSt (and their possible isomers). From the practical point of view which was stated before, monounsaturated species are the most important. Supplementary Fig. 5A and B shows the profiles of this species type: POP, PLP, POSt, StOSt, StLSt, and PLSt (the notation might not represent the position of the respective acyl moiety in the glycerol backbone). Monounsaturated species showed a linear rate of rising up to 12 h and a plateau was observed after that time at 60 °C, whereas at 50 °C it continued to increase but at a slower rate. The predominant species was PLSt, which reached a content of 9.36% and 11.85% at 50 and 60 °C, respectively, at the final reaction time. This result was as expected from a probabilistic point of view based on the possible reactions involved between original TAG in SO and palmitic and stearic acids. In general, the remaining species content in decreasing order of weight were: PLP > POSt > StLSt ~ POP > StOSt for both tested temperatures. The linoleic acid was the predominant unsaturated fatty acid in SUS species. This is related to the fact that it is the major fatty acid in original oil. Moreover, it is the predominant fatty acid in the TAG *sn*-2 position (64.63%).

In reference to trisaturated species (see Supplementary Fig. 6A and B), PPSt and StStP species were predominant. This result was in agreement with the possible acyl migration reactions involved between disaturated TAG majority and palmitic and stearic acids. The amount of these species in reaction products became important after 12 and 24 h of reaction at 60 °C and 50 °C, respectively. These times correspond to conditions in which a larger amount of diacylglycerols were available to be esterified.

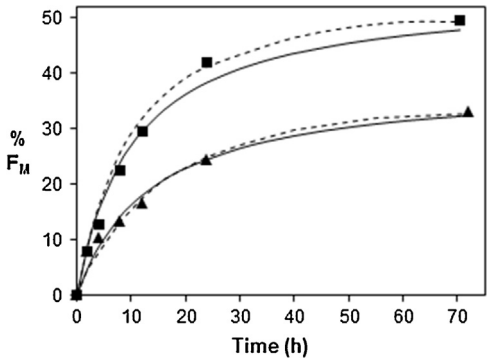

Fig. 4. Incorporation of P + St into SO during acidolysis as a function of time and temperature (50 °C = ▲ and 60 °C = ■): fitting of experimental values by kinetic Model I (–) and Model II (···).

Values shown in this section were part of the experimental data used for obtaining Model III and IV parameters. Furthermore, glycerol was not found in any of the reaction products, indicating that hydrolysis was never completed.

3.3.3. Mathematical modeling of acidolysis kinetics with immobilized lipases

Experimental data were fitted with the aforementioned models. Results obtained in each case are shown below. Modified Model I was not required for modeling acidolysis reaction catalyzed by immobilized lipases since no lag period was observed in either tested reaction temperatures.

3.3.3.1. Kinetic Model I. The usage of the Kinetic Model I meant the fitting of experimental data (P + St incorporation into acylglycerides vs. time) through Eq. (1). For both temperatures, fittings succeeded in describing the experimental points properly (Fig. 4), taking into account the values of the determination coefficient. The associated parameters, F_{Me} and $t_{1/2}$, proved to have dependence on temperature (Table 3). As it can be seen, F_{Me} increased significantly, while $t_{1/2}$ decreased with the temperature increment. The maximal incorporation achievement at 60 °C with the tested biocatalyst was very similar to the one obtained by Pacheco et al. [10] using a commercial one, Lipozyme RMIM, for the same substrates but in other molar and dilution ratios. However, parameters obtained in this work, F_{Me} and $t_{1/2}$, were more temperature-dependent and higher, respectively, than those reported in the last work. This means that although the final equilibration incorporation was similar (at 60 °C), the reaction presented here proceeded at a lower velocity requiring higher times to reach similar incorporation levels. This behavior is in agreement with the fact that in this contribution a lower FFA:oil ratio and a higher amount of solvent was used.

By comparing previous parameters to the obtained ones for modeling of the lipase activity in its free status (Table 2), at 50 °C the expected F_{Me} resulted very similar (40.85% for the free lipase), but not so comparable for $t_{1/2}$ value (19.075 h for the free lipase). On the other hand, results obtained at the optimum temperature assayed for the immobilized lipase, 60 °C, can be compared to the ones obtained at the optimum temperature for the free lipase, 40 °C, which indicates that equilibrium incorporations were similar

Table 3
Parameters of Model I (Eq. (1)) for acidolysis with immobilized *Rhizomucor miehei* lipases at assayed reaction temperatures and the corresponding determination coefficients.

T (°C)	F_{Me} (mol%)	$t_{1/2}$ (h)	R^2
50	38.547	13.654	0.9569
60	54.740	10.321	0.9813

Table 4
Parameters of Model II (Eq. (2)) for acidolysis with immobilized *Rhizomucor miehei* lipases at assayed reaction temperatures and the corresponding determination coefficients.

T (°C)	A (mol/(g h))	B	C	K_e	R^2
50	1.56E−05	2.7124	1.0004	0.4937	0.9308
60	2.93E−05	1.7922	0.9999	2.4034	0.9981

(50.27% for the free lipase), whereas the $t_{1/2}$ values were different (6.194 h for the free lipase). This $t_{1/2}$ values difference could be related to the lag period subtracted in the free lipase case in order to be able to apply Model I. Therefore, it can be affirmed that F_{Me} was not affected by the immobilization process when the reaction conditions were optimal. Moreover, the immobilization process improved the catalytic performance of free lipases avoiding the lag phase. It is worth mentioning that the amounts of protein were different in each case, because in each occasion it was tried to achieve good incorporations. So, a lesser amount of lipase was used in its free state than in its immobilized form in order to avoid formation of inactive aggregates [40].

3.3.3.2. Kinetic Model II. Required values from the derived function involved in the equation to adjust (Eq. (2)) were calculated from the obtained expression with the Model I for F_M. Parameters resulted from Model II fitting, A, B, C, and K_e, are shown in Table 4. By comparing these parameters with those reported by Camacho et al. [11] for the acidolysis of triolein and capric acid at 30 °C (A = 0.00782 mol/g h, B = 2.17 mol/g h and K_e = 1.26) and with those reported by Pacheco et al. [10] for the acidolysis under the conditions mentioned above at temperatures between 40 and 60 °C (A = 0.0038–0.0089 mol/g h, B = 0.53–3.68 mol/g h, and K_e = 0.64–1.42), it was possible to notice what is stated below. Values found in the current system for the parameter A were much smaller than those reported in both studies, while values of the parameters B and K_e were within the range of variation of the reported corresponding ones. Values of parameter B (K_L/K_M) were slightly higher than one, which indicates a little more affinity of lipases for the native fatty acids than for P + St. The parameter C (k_L/k_M) remained very close to 1, which indicates that the reaction between diacylglycerols and the acyl-enzyme complex was very fast and temperature did not affect the probability of contact between them. This result was consistent with that reported by Camacho et al. [11] and Pacheco et al. [10]. Based on the above, it could be concluded that the parameter A in Model II (involving the active enzyme concentration) would be the most affected by the reaction conditions. It is worth mentioning that the value of the active enzyme concentration in each compared system is unknown, so this could possibly be the more important factor affecting the A value.

Fig. 4 shows in dotted lines the F_M profiles as time function, obtained by numerical integration of the Eq. (2). As it can be observed, the lumped parameters model represented successfully the reaction system analyzed when lipases immobilized on modified chitosan modified microspheres are used as catalysts. Moreover, the fittings were adequate unless not all the original hypothesis were fulfilled – DAG concentration did not remain at low level during all the reaction time – and the acyl migration as side reaction was not considered in the model – the presence of SSS in the reaction products indicated that this side reaction took place in the studied system –.

3.3.3.3. Kinetic Model III. Experimental data of the molar concentration of palmitic and stearic free fatty acids reagents (S) in the reaction medium depending on time are showed in Fig. 5 along with the predictions obtained through Model III. Since this model served

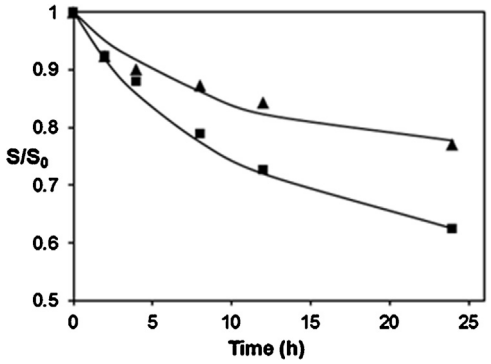

Fig. 5. Concentration of saturated fatty acids in the reaction medium respect to the initial concentration (S/S_0) as a function of time and temperature (50 °C = ▲ and 60 °C = ■): fitting of experimental values by kinetic Model III.

to describe properly the behavior up to the hour 24 of reaction only this time range is shown. The fitting parameters are indicated in Table 5, and as it can be seen the high values of determination coefficients obtained show that Model III succeeded in describing accurately the experimental data for the specified time range. The ratio between obtained values of V_{R1} and V_{M2} parameters (V_{R1}/V_{M2}) tended to increase with temperature (from 1.45 at 50 °C to 1.85 at 60 °C), quite similar to that reported by Pacheco et al. [10]. This implies that the increase of temperature favors the rate of the esterification reaction respect to that of the hydrolysis one. On the other hand, K_{II} value also increases with temperature (300 and 1850 at 50 and 60 °C, respectively), revealing that the formation of the complex between intermediary compounds and the enzyme is favored with this increment. K_{II} values are superior to those reported by Pacheco et al. [10]; this difference is probably due to the fact that the reaction conditions and the characteristics of the biocatalyst used in both studies are different.

On the other hand, the fact that Model III was not able to represent the actual system behavior around all the range of tested times (specifically at more than 24 h) might be associated to the fact that some of the assumptions become invalid for longer times. It could be occurring that the rate determining step is no longer the one that involves the rupture of the ester bond, but another related to the saturated fatty acids availability. However, high DAG and SSS levels reached after 24 h of reaction caused that extending the reaction for longer time does not result suitable from a practical point of view.

3.3.3.4. Kinetic Model IV. This proposed model allows representing the variation of different species of TAG, DAG and FFA, grouped according to their saturation level, over reaction time. It is said: TAG were classified as SSS, SUS, UUS, and UUU, DAG were classified as UU− and −US, and FFA as U and S. Experimental DAG composition was not obtained as different groups, so −US and UU− model variables were added to fit experimental DAG data. Experimental and simulated data are represented in Fig. 6. Kinetic parameters and global correlation coefficients of Model IV are summarized in Table 6. It could be observed that this proposed model fit satisfactorily the experimental data in both tested temperatures (showing p-values for the lack of fit (LOF) test higher than

Table 5
Parameters of Model III (Eq. (5)) for acidolysis with immobilized *Rhizomucor miehei* lipases at assayed reaction temperatures and corresponding determination coefficients.

T (°C)	$V_{R1}E_T$ (1/h)	$V_{M2}E_T$ (1/h)	K_{II} (1/M)	W_0 (M)	R^2
50	2.180	1.5	300	0.067	0.994
60	14.500	7.8	1850	0.072	0.969

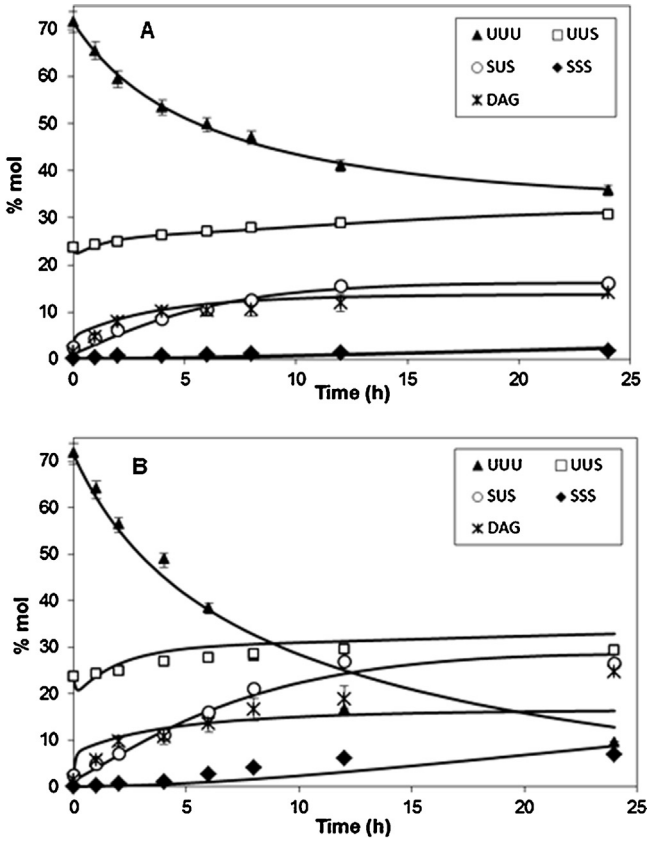

Fig. 6. Evolution of triacylglycerols groups and diacylglycerols: fitting of experimental values by kinetic Model IV. (A) 50 °C and (B) 60 °C.

the significance level of 0.05). From the kinetic constant values obtained, it could be concluded that the reaction toward the formation of UU− diacylglycerols is most favored by the increment in temperature than the −US formation (k_1 value increased near 70% and k_{-1} value decreased in 2 magnitude orders when temperature change from 50 to 60 °C; however, k_3 and k_{-3} values changed to a lesser proportion). From the point of view of the formation of TAG because of the incorporation of a saturated fatty acid into a DAG, it could be seen that the enzymatic system showed a preference to incorporate S into −US rather than into UU−. This could be related to a steric hindrance caused by the tridimensional conformation adopted by acylglycerides when unsaturated fatty acids are present in their structure. With regard to the formation of SUS,

Table 6
Parameters of Model IV (Eqs. (11)–(19)) for acidolysis with immobilized *Rhizomucor miehei* lipases at assayed reaction temperatures and corresponding determination coefficients.

Kinetic parameters (1/(h M))	Temperature (°C)	
	50	60
k_1	3.574E−08	6.033E−08
k_{-1}	5.702E−09	2.157E−11
k_2	1.741E−08	1.300E−08
k_{-2}	4.160E−07	6.951E−07
k_3	6.974E−08	9.204E−08
k_{-3}	2.045E−09	8.837E−10
k_4	4.890E−08	4.302E−08
k_{-4}	1.176E−05	7.833E−06
k_5	2.824E−09	6.640E−09
W_0 (M)	0.371	0.371
R^2	0.9841	0.9362
p(LOF)	0.181	0.184

although absolute values of kinetic constants related to this forward and reverse reaction (k_4 and k_{-4}) decreased by the increment in temperature, k_{-4} turned out to be the most modified. This effect over kinetic constants favored the displacement to the formation reaction adding up to the higher DAG concentration.

Furthermore, an acceptable fitting of SSS appearance related to acyl migration reaction was obtained. As it can be observed in Table 6, the related kinetic constant, k_5, was highly influenced by the increment in temperature.

3.3.3.5. Models validation. A validation analysis was performed in order to examine the robustness of models. Acidolysis reactions were performed at 60 °C, varying the amount of biocatalyst (between 0.30 and 0.39 g), the initial P + St:SO molar ratio (between 3 and 6) and the amount of hexane (from 1.6 to 3.2 ml). Experimental results were compared to simulated values obtained using the corresponding parameters to each model. When enzyme concentration was changed, values of kinetic constants (except k_5) in Model IV were recalculated considering that apparent kinetic constants had a linear dependency with the enzyme concentration. Percentage root mean squared error (%RMSE) values were 1.73, 13.30, 5.30, and 2.23 to Models I, II, III, and IV, respectively. A good predictive capacity was showed by Models I, IV, and III in this order. However, a big difference between them is that Models I and III are able to predict only one system response while Model IV let know the behavior of 5 responses simultaneously, making it more useful. It is worth mentioning that parameters of these models were obtained and validated using a non-homogeneous TAG refined oil (with a high content of oleic and linoleic acids) and a mixture of saturated fatty acids (palmitic and stearic acids). Model IV only takes into account differences between saturated and unsaturated fatty acids, but not the level of their unsaturation (as mono- or diunsaturated fatty acids) or their chain length. Thus, some deviations may be expected from model predictions if pure TAG, other oils and/or another free fatty acid are used. Moreover, the presence of some oil minor components could also modify the obtained behavior. With this in mind, this model could be used as a valid and practical tool to predict the expected composition of products when refined sunflower oil is enzymatically modified by acidolysis using palmitic and stearic acids as acyl donors and the new immobilized biocatalyst.

4. Conclusions

In this study, the kinetic behavior of the acidolysis reaction of sunflower oil with saturated fatty acids using a particular biocatalyst prepared by immobilizing lipases from *R. miehei* on modified chitosan microspheres was investigated. Among the most important results are highlighted that the formation of diacylglycerols was greater than the monoacylglycerols generation, and the hydrolysis was never complete because glycerol was not detected in the reaction products. Also, the increased reaction temperature generated major changes in the overall composition of acylglycerols and gave rise to the highest composition of palmitic and stearic acids in the obtained structured lipids (58% after 70 h at 60 °C).

On the other hand, to represent the evolution of acidolysis over time four kinetic models of different complexity were tested (three of them developed by other authors and the last proposed in this work), which achieved to fit the experimental data very successfully. In the specific case of Model I, kinetics parameters could be compared with those found in modeling of acidolysis performed by free *R. miehei* lipases. The results indicated that the immobilization process improved the catalytic performance of free lipases avoiding the lag phase. Furthermore, the maximal incorporation of saturated fatty acids achieved at 60 °C with the tested biocatalyst was very similar to the one obtained with free lipases at optimal temperature and with Lipozyme RMIM (a commercial immobilized lipase) in a previous contribution. Regarding Model II, it was able to successfully describe the experimental behavior but it did not allow an accurate prediction when reaction conditions were changed. With respect to Model III, it only achieved describing adequately the kinetic behavior up to the hour 24 of reaction. This would indicate that some hypotheses of the model were not valid any more and/or other non-considered reactions in that model were relevant from that time. The formation of trisaturated triacylglycerols, SSS, during the experimental reactions is expected to be the result of a non-enzymatic side reaction, the acyl migration one, which was not taken into account in that model. Model IV, proposed in this work, enhance the predictive capacity of previous models allowing to obtain more detailed information about the behavior of TAG, DAG, and FFA groups (according to their saturation level) and the formation of SSS related to the acyl migration reaction. This model provided very valuable information about single reaction patterns since individual kinetic constants of the proposed first order kinetic rates were successfully obtained. Moreover, the influence of the enzyme concentration could be well established by a linear relation with the corresponding apparent kinetic constant validating the model performance in other conditions. However, fatty acid specificity was not considered in the proposed model. Therefore, the goodness of its performance must be checked when other oils or free fatty acids are going to be used.

In this way, the benefits of having parameters of each model not only allow comparison with data from similar systems obtained in other scientific works, but also its implementation in future applications of design, optimization and process control. Moreover, information obtained by Model IV could be used to set up the desired compositional quality in the structured lipids produced by enzymatic acidolysis. The composition of trisaturated triacylglycerols could be used as an additional parameter to establish the final reaction time.

Conflict of interest

All authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2014.06.006.

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